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Plasma immunological markers in pregnancy and cord blood: A possible link between macrophage chemoattractants and risk of childhood type 1 diabetes

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Abstract

Problem: Previous studies have suggested that immune perturbations during pregnancy can affect offspring type 1 diabetes (T1D) risk. We aimed to identify immunological markers that could predict offspring T1D or that were linked to T1D risk factors.

Method of study: We quantified selected circulating immunological markers in mid-pregnancy (interleukin (IL)-1 β , IL-1 receptor antagonist (IL-1Ra), IL-2 receptor α (IL-2Ra), IL-2, -4, -5, -6, -10, -12p70, -13, -17A, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)- γ , C-X-C motif chemokine 10 (CXCL10, interferon gamma-induced protein 10), C-C motif chemokine ligand (CCL) 2 (monocyte chemo-attractant protein 1), CCL3 (macrophage inflammatory protein (MIP) 1 α), CCL4 (MIP-1 β), tumor necrosis factor (TNF)) and cord blood plasma (neopterin and kynurenine/tryptophan ratio) in a case control-study with 175 mother/child T1D cases (median age 5.8, range 0.7–13.0 years) and 552 controls.

Results: Pre-pregnancy obesity was positively associated with CCL4, CXCL10, kynurenine:tryptophan-ratio, and neopterin ($p < 0.01$). The established T1D SNPs rs1159465 (near *IL2RA*) and rs75352297 (near C-C chemokine receptor (*CCR*)2 and *CCR3*) were positively associated with soluble IL-2R α and CCL4, respectively ($p < 0.01$). There was a borderline association of CCL4 and offspring T1D risk, independent of maternal obesity and genotype. When grouping the immunological markers into M1, M2, Th1-, Th2-, or Th17 cell phenotypes, there was a borderline association ($p = 0.02$) with M1 phenotype and no association between M2-, Th1-, Th2-, or Th17 phenotypes.

Conclusions: Increased mid-pregnancy CCL4 levels showed borderline associations with increased offspring T1D risk, which may indicate a link between environmental factors in pregnancy and offspring T1D risk.

Keywords: Cord Blood, Cytokine, Human, Inflammatory markers, Norwegian Mother and Child Cohort Study, Pregnancy, Type 1 diabetes.

1 INTRODUCTION

2 Type 1 diabetes (T1D) is one of the most prevalent chronic conditions in children, and is
3 associated with increased morbidity and mortality ¹. Mechanisms driving the rising incidence
4 in most countries over past decades are unknown. The insulin-producing pancreatic β -cells
5 are destroyed in an autoimmune process, triggered or driven by environmental and genetic
6 factors increasing T1D susceptibility ², which may operate already in fetal life ³.

7 Cytokines are small proteins secreted by nearly all cell types to induce or modulate
8 immune responses, and play important roles in a wide spectrum of pathologies. Cytokines
9 are pleiotropic, and combinations of cytokines can dictate T-helper (Th) lymphocyte
10 differentiation, or activate macrophages to pro- and anti-inflammatory states ^{4,5}, immune cell
11 phenotypes that secrete distinct cytokine repertoires. Several T1D susceptibility genetic
12 markers are in or near genes associated with cytokines or cytokine signaling ⁶. Cytokine-
13 driven immune responses are likely factors leading to β -cell destruction ⁷, and evidence is
14 accumulating that circulating cytokines and related markers may predict T1D ^{8,9}.

15 Cytokines are also involved in regulating normal pregnancy ¹⁰. Pregnant women with
16 an inflammatory state involving cytokines such as infections, obesity ¹¹ and higher age have
17 a higher risk of offspring T1D ^{3,12}. Maternal cytokine secretion may affect the fetus for
18 instance by inducing inflammation in, or crossing over, the placenta. Although some
19 cytokines such as IL-6 can cross the placenta ¹³, most cytokines and inflammatory markers
20 do not ^{13,14}. However, macrophage accumulation ¹⁵ and expression of toll like receptors in
21 the placenta may amplify maternal inflammatory signals ¹⁶, and thus contribute to fetal
22 disease risk ¹⁵. Previous studies have reported associations between maternal inflammatory
23 markers and later health outcomes such as asthma and allergies ¹⁷, supporting that maternal
24 inflammatory cytokines affect the offspring disease susceptibility later in life. To the best of
25 our knowledge, there is only one small study published on maternal cytokines and offspring
26 T1D risk. This study reported an association between increased early-pregnancy serum
27 levels of IFN- γ and IL-1 β and higher risk of offspring T1D ¹⁸; however, such associations
28 need to be confirmed in larger studies.

29 In order to address the knowledge gap regarding antenatal immune perturbations
30 associated with offspring T1D risk, we aimed to study circulating immunological markers in
31 maternal mid-pregnancy plasma (interleukin (IL)-1 β , IL-1 receptor antagonist (IL-1Ra), IL-2
32 receptor α (IL-2Ra), IL-2, -4, -5, -6, -10, -12p70, -13, -17A, granulocyte-macrophage colony
33 stimulating factor (GM-CSF), interferon (IFN)- γ , C-X-C motif chemokine 10 (CXCL10,
34 interferon gamma-induced protein 10), C-C motif chemokine ligand (CCL) 2 (monocyte
35 chemo-attractant protein 1), CCL3 (macrophage inflammatory protein (MIP) 1 α), CCL4 (MIP-
36 1 β), tumor necrosis factor (TNF)) and markers of IFN- γ activation in the child's cord blood
37 plasma (neopterin and the kynurenine to tryptophan ratio (KTR)), in a large case-control

1 study of offspring T1D, nested within one of the largest pregnancy cohorts in the world. The
2 immune markers measured in maternal plasma were carefully selected, either due to prior
3 association to T1D or celiac disease, to ensure representation of several types (Th1, Th2,
4 Th17, and Treg) of immune responses, and believed to give reliable measurements given
5 our sample handling. As cord blood plasma were not immediately separated and more
6 frequently are affected by some degree of hemolysis we measured only neopterin and KTR
7 in cord blood, two inflammatory markers believed to be relatively stable during sample
8 handling and storage ¹⁹. A secondary aim was to assess associations of other suggested risk
9 factors for type 1 diabetes, such as maternal obesity or genotype, and these circulating
10 immunological markers.

METHODS

Subjects and design

We designed a nested case-control study in the Norwegian Mother and Child Cohort Study (MoBa)²⁰. MoBa recruited around 114,000 pregnancies from all over Norway during 1999-2008, and children who developed T1D by February 5, 2014 were identified with a high degree of ascertainment by register linkage to the Norwegian Childhood Diabetes Registry²¹. In all, 175 mother/child-pairs were included as T1D cases, and 552 mother/child-pairs from a random sample of the cohort with available blood samples were used as controls (Figure 1). The randomly selected controls were largely similar to the whole MoBa cohort, with the exception of caesarean section and premature birth (15.2% vs 10.3% for caesarean section, and 6.2% vs 3.4% for premature birth, in the whole MoBa cohort vs picked random controls)²², conditions associated with missing blood samples in the MoBa Cohort. Characteristics of the study participants are given in Table 1. The establishment and data collection in MoBa has obtained a license from the Norwegian Data Inspectorate and approval from The Regional Committee for Medical Research Ethics, and the study was approved by The Regional Committee for Medical Research Ethics in South-Eastern Norway. Informed consent was obtained from all study participants.

Sample handling

Venous maternal blood were collected in EDTA tubes around pregnancy week 17-19, centrifuged and shipped overnight as plasma in ambient temperature to the Norwegian Institute of Public Health for storage at -80 °C. At delivery, umbilical cord vein blood were collected in EDTA tubes and shipped overnight in ambient temperature before centrifugation and storage at -80 °C until analysis. Cord blood plasma samples were visually assessed for degree of hemolysis and graded according to a four level color code²³. Maternal and cord blood samples were treated separately but equally, with personnel blinded to case/control status at all stages of sample handling and analysis.

Laboratory assays

We selected a limited set of cytokines based on prior association to either celiac disease or T1D, and to ensure representation of macrophage M1 and M2 phenotypes, and Th1, Th2, Th17, and Treg immune responses in the adaptive immune system. We avoided cytokines known or suspected to be present in undetectable concentrations in most healthy individuals, or to give unreliable measurements e.g. due to pre-analytical factors. In total we measured 18 cytokines in maternal mid-pregnancy plasma using Bio-Plex protein array systems (Bio-Rad, Hercules, CA), based on xMAP technology (Luminex, Austin, TX) as previously described²³ – interleukin (IL)-1 β , IL-1 receptor antagonist (IL-1Ra), IL-2 receptor α (IL-2Ra),

IL-2, -4, -5, -6, -10, -12p70, -13, -17A, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)- γ , C-X-C motif chemokine 10 (CXCL10, interferon gamma-induced protein 10), C-C motif chemokine ligand (CCL) 2 (monocyte chemo-attractant protein 1), CCL3 (macrophage inflammatory protein (MIP) 1 α), CCL4 (MIP-1 β), tumor necrosis factor (TNF)). Concentrations were calculated from a standard curve generated from standards provided by the assay manufacturer, and standard curves showing high sensitivity were selected to obtain similar lower limit of quantification (LLOQ) for all samples. The LLOQ ranged from 0.02 (IL-4) to 1.35 (CCL2) pg/mL, and corresponds to the lowest point of the standard curve yielding acceptable recovery. The inter-plate mean coefficient of variation (CV) across all dilutions ranged from 2.2% (CCL4) to 8.0% (IL-4). Further details of limits of quantification, inter- and intra-assay CVs are given in previous publication²³. Umbilical-cord plasma concentrations of cotinine, neopterin, tryptophan and kynurenine were measured using a high-throughput liquid chromatography tandem mass-spectrometry at the Bevitall laboratory (Bergen, Norway)²⁴, which have previously demonstrated acceptable pre-analytical stability²⁵. The kynurenine/tryptophan ratio (KTR) was calculated by dividing the plasma concentration of kynurenine (nmol/L) by the concentration of tryptophan (μ mol/L). A brief summary of the immune markers studied is given in the online repository of Mårild et al.²³.

Genotyping

To account for established T1D susceptibility markers, participants were genotyped using a custom Illumina Golden Gate assay (Illumina, San Diego, CA). DNA extraction, genotyping methods and quality control procedures are described in detail in previous publication²³. Briefly, tag-SNPs (n = 144) on chromosome 6 were used to impute human leukocyte antigen (HLA) class II genotype²⁶, which was subsequently confirmed by classical HLA genotyping using allele specific PCR. Based on the previously established risk of T1D conferred by HLA genotype, we categorized genotypes into four groups: protective (carrying at least one copy of HLA DQA1*01:02-DQB1*06:02-DRB1*15:01 [DQ6-DR15]), increased risk (at least one copy of HLA DQA1*03-DQB1*03:02-DRB1*04 [DQ8-DR4] or DQA1*05:01-DQB1*02:01 [DQ2-DR3], but not both haplotypes), high risk (heterozygous HLA DQ2-DR3/DQ8-DR4) or neutral (any other genotype). Established non-HLA T1D risk SNPs (n=51) were used to calculate a weighted additive genetic risk score (GRS, sum of risk alleles, weighted by their odds ratio (OR) for T1D reported in the literature, see Table S1 in the Online Repository). SNPs possibly associated with T1D and cytokine levels (n = 19, see Table S2 in the Online Repository) were tested against maternal immunological markers to assess if there were any specific genetic effects that should be included in the analysis.

Other variables

Predefined variables potentially associated with the selected immunological markers and/or T1D were assessed as predictors for the immunological markers and included as covariates in the analysis of association between cytokines and T1D. Maternal smoking was assessed by self-reported questionnaires and concentration of umbilical cord plasma cotinine²⁴ (a biomarker for recent nicotine exposure, with children having ≥ 30 nmol/L assumed to be exposed to cigarette smoke during pregnancy irrespective of maternal self-reported data or missing information). Maternal infection frequency was assessed from self-reported questionnaires²³. Maternal age at delivery, parity, mode of delivery, child's sex, birth weight, gestational age, month and calendar year of birth were collected from the Medical Birth Registry of Norway. Pre-pregnancy body mass index (BMI) was collected from both questionnaires and birth records, and obesity defined as BMI ≥ 30 . Maternal T1D was collected from birth records, self-reported questionnaires and ICD-10 codes E10.0-E10.9 in the Norwegian Patient Registry. The distribution and categorization of these variables are shown in Table 1. The number of freeze thaw cycles (median 4, range 1 – 10 for maternal samples and median 3, range 1 – 9 for cord blood samples) had a relatively small, but statistically significant association with several cytokine concentrations, while degree of hemolysis in cord blood had a similarly small, but significant effect on neopterin²³. Maternal samples were spun down at the hospital then shipped overnight (median 1 day in transit, interquartile range (IQR) 1 – 2 days), while cord blood samples were shipped overnight then spun down at arrival (median 2 days in transit, IQR 1 – 3 days). Time in transit had a small, but significant effect on neopterin (fold difference 1.04, $p < 0.01$), but we did not adjust for time in transit as the effect on the reported neopterin estimates was negligible.

Statistical analysis

A priori, we decided to base our primary analysis on a logistic regression model with T1D as the dependent variable and log₂-transformed immunological markers as the explanatory variables. In a secondary analysis, we assessed possible deviation from linearity by testing the significance of a squared term of the explanatory variables and by categorical analysis. Based on the distribution of the immunological marker among controls, observations were categorized into tertiles. For markers where $>33\%$ of samples were below detection limit (IL-1 β , IL-5, IL-12p70, IL-13, IFN- γ , CCL2, IL-2R α , IL-17A), all the values below the detection limit were put in the lowest category and remaining levels were split in two groups at their median. Associations between predictors (maternal BMI, etc) and immunological markers in pregnancy and cord blood were assessed using linear regression with the log₂-transformed immunological markers as the dependent variable. As 20 immunological markers with some a priori evidence for association with T1D were tested, and cytokines tend to be positively correlated, Bonferroni correction for multiple testing would

1 be too conservative ²⁷. We decided a priori to use 0.01 as our significance level in the
2 analyses of each single immunological marker.

3 The primary statistical test of the association between immunological markers and the
4 offspring's risk of T1D was a $m-1$ degree of freedom likelihood ratio test, where m equals the
5 number of immunological markers (testing the complete null hypothesis that none of the
6 markers were associated vs the alternative that at least one marker was associated with
7 offspring T1D risk). Next, we used a likelihood ratio test to assess each of five subsets of
8 immunological markers grouped as M1-, M2-, Th1-, Th2-, or Th17-pathway immunological
9 markers (see Figure 2, Panel A for a simplified overview of pathways and their cytokines).
10 We attempted to choose the most characteristic immunological markers for these subsets,
11 and tried to avoid including immune markers which overlap between different subsets, thus
12 not all immunological markers shown in the figure are included. Z-scores for each cytokine
13 were calculated and used in the analysis of groups and the model including all cytokines.
14 Further, we tested each single immunological marker as predictors of T1D, adjusted for
15 maternal pre-pregnancy BMI, child's HLA risk category, gestational age at delivery, mode of
16 delivery and year of birth. We also assessed the impact of additional adjustment for maternal
17 smoking during pregnancy, child's non-HLA genetic risk score, maternal T1D, parity, freeze-
18 thaw cycles, and maternal age. Finally, we did an exploratory, hypothesis free analysis of
19 cytokine patterns using k-means cluster analysis with 8 clusters on the log2-transformed
20 maternal immunological markers. This assigned individuals to clusters (groups) with similar
21 pattern across the 18 maternal immunological markers. We then compared the resulting
22 clusters with respect to risk of offspring T1D in a logistic regression model.

RESULTS

Established risk factors for T1D such as HLA class II and maternal T1D were more common in cases than in controls. Other characteristics were similar in cases and controls, except a higher proportion of maternal BMI above 30 among cases was observed (see Table I). The child's median age at the end of follow-up was 8.6 (range 4.7 – 14.1) years in controls (see Table I), and the median age at diagnosis of T1D cases was 5.8 (range 0.7 – 13.0) years. The distribution of most immunological markers were approximately symmetrical after log₂-transformation (see Figure S1 in the Online Repository), although for around half of the markers a relatively large proportion of the participants had levels below the lower limit of quantification. There were strong positive pairwise correlations between immunological markers measured in mid-pregnancy maternal plasma (IL-1 β , -1Ra, -2Ra, IL-2, -4, -5, -6, -10, -12p70, -13, -17A, GM-CSF, IFN- γ , CXCL10, CCL2, CCL3, CCL4, TNF), but not in markers measured in cord blood plasma (neopterin, KTR) (see Figure S2 in the Online Repository). Maternal mid-pregnancy IFN- γ was not associated with child's neopterin or KTR.

Predictors of immunological markers

There was no significant seasonal variation for the level of any of the immunological markers, which implies seasonal infections do not influence levels of the immunological markers in a systematic fashion (See Figure S3 in the Online Repository). Maternal pre-pregnancy BMI was positively associated with maternal CCL4 and CXCL10 concentrations, as well as neopterin and KTR in cord blood, and had a borderline ($p = 0.02$) positive association with IL-6 (see Table S3 and Figure S4 in the Online Repository). Notably, maternal BMI significantly predicted cord blood neopterin and KTR also after adjusting for the child's birth weight (or ponderal index), while birthweight did not significantly predict cord blood concentrations of neopterin and KTR when adjusted for mothers BMI. Maternal levels of GM-CSF were lower in preterm deliveries and increased in mothers smoking during pregnancy. Carrying the T1D high-risk HLA genotype (heterozygous DQ2/DQ8) was associated with cord blood KTR (see Table S3 and Figure S4 in the Online Repository). The T1D non-HLA genetic risk score was not significantly associated with any of the immunological markers (see Table S3 in the Online Repository). Some maternal SNPs were associated with maternal mid-pregnancy cytokine levels at $p < 0.01$, such as *IL2RA* SNP rs11594656²⁸ associated with soluble IL-2R α , SNP rs3024505 (flanking *IL10*) associated with IL-10 and SNP rs75352297 (intergenic between C-C chemokine receptor (*CCR*)3 and *CCR2*) associated with CCL4 (see Table S2 and Figure S4 in the Online Repository).

Pregnancy and cord blood immunologic markers and risk of type 1 diabetes

The overall likelihood ratio test of the null hypothesis that none of the 20 immunological markers (IL-1 β , -1Ra, -2Ra, IL-2, -4, -5, -6, -10, -12p70, -13, -17A, GM-CSF, IFN- γ , CXCL10, CCL2, CCL3, CCL4, TNF measured in maternal mid-pregnancy plasma, and neopterin and KTR measured in cord blood plasma at birth) were associated with T1D gave $p = 0.17$. The likelihood ratio tests of the macrophage M1 (CCL2, -3, -4, CXCL10, IFN- γ , GM-CSF) immunological markers gave $p = 0.02$, and the related M2 (IL4, IL13, IL1ra, IL10) immunological markers gave $p = 0.13$, while tests for the Th1, Th2 and Th17 groups gave $p \geq 0.20$ (see Figure 2B).

Modelling each single immunological marker for association with T1D revealed no significant associations at the predefined 0.01 level after adjustment for covariates. Maternal mid-pregnancy plasma CCL4 showed a borderline association with T1D per doubling ($p = 0.04$ per log₂ increase, see Figure 3). Some immunological markers had an increased estimate and borderline association (ORs between 1.52 and 1.60, p -values between 0.02 and 0.05) for the highest tertile category in the unadjusted analysis, such as maternal mid-pregnancy plasma CCL2, IFN- γ , IL-4 and TNF, but these were all not significant after adjustment although their estimates were still increased (data not shown, and see Figure S5 in the Online Repository). Adjustment for potential confounders had limited influence on most associations (see Figure S6 in the Online Repository). Likewise, adjusting for genotypes influencing immunological marker levels (e.g. adjusting for the rs75352297 genotype, which was significantly associated with CCL4 levels, when analyzing CCL4) did not influence our results to an appreciable degree (data not shown). Of note, the association between maternal obesity (BMI ≥ 30) and higher risk of offspring T1D (OR 2.28, 95% CI: 1.35 – 3.85, compared to BMI < 25) remained significant after adjustment for immunological markers CCL4, CXCL10, neopterin and KTR (adjusted OR 1.99, 95% CI: 1.09 – 3.64).

Posthoc analyses

For immunological markers where there was a suggestive threshold association with T1D at the highest category (CCL4, IFN- γ , IL17a, IL2R α , TNF, neopterin, see Figure S5 in the Online Repository), we explored associations using the 90th percentile as threshold. CCL4 (OR 2.37, 95% CI 1.10 – 5.10, $p = 0.03$), neopterin (OR 0.40, 95% CI 0.16 – 0.99, $p = 0.05$) and IL17a (OR 2.16, 95% CI 1.03 – 4.54, $p = 0.04$) had suggestive associations with T1D development, but these must be interpreted with caution as the analyses were data-driven.

As a sensitivity analysis, we adjusted for celiac disease (present in 21 cases and 5 controls), which only slightly changed our estimates, with the largest change in the adjusted estimate per log₂ increase of CCL4 and T1D risk (OR 1.52, 95% CI 1.08 – 2.13, $p = 0.02$).

Finally, k-means cluster analysis on the maternal cytokines reliably clustered individuals in eight groups based on similarity in pattern of cytokine concentrations. Visual

1 inspection of patterns of mean cytokine concentrations across the eight groups did not have
2 any obvious correspondence to any of our a priori pathways (Th1, M1, etc.), and there was
3 no clearly significant association between assigned group and risk of T1D, with the exception
4 of cluster 8 which had a borderline protective association (OR 0.52, 95%CI 0.29 –0.92, $p =$
5 0.03) when compared to Cluster 1. Cluster 1 was characterized by high levels of all
6 cytokines, in particular IFN- γ and IL-17a, while cluster 8 was characterized by average and
7 slightly higher than average level of all measured maternal immune markers except CXCL10,
8 CCL4 and IL2-R α , which were slightly lower than average (Figure S7).

1 Discussion

2 While none of the immunological markers were significantly associated with
3 childhood-onset T1D according to our predetermined 0.01 level, there were borderline
4 significant associations of increased maternal CCL4 with childhood T1D risk. Another finding
5 was that maternal pre-pregnancy obesity was associated with higher concentrations of
6 CCL4, CXCL10, neopterin and KTR, and remained associated with T1D also after
7 adjustment for these immunological markers.

8 Interestingly, a common feature of CCL4 and other cytokines with a possible
9 association to T1D risk in the offspring, such as CCL2 and IFN- γ , is activation of pro-
10 inflammatory macrophages. In response to tissue damage and pathogen invasion, CD4+ T-
11 cells are differentiated to Th1, which main product is IFN- γ . IFN- γ stimulates macrophages to
12 become pro-inflammatory ²⁹, designated M1 macrophages known to secrete chemokines like
13 CCL4 and -2 ³⁰, as opposed to M2 macrophages that are anti-inflammatory and a result of
14 stimulation by IL-4 and -10 and with a different secretory repertoire. Pregnancy is dependent
15 on a finely tuned regulation of immune responses, and after the first trimester the maternal
16 circulating cytokine pattern skews towards a type 2 polarization ³¹, while IFN- γ levels are
17 depressed throughout the whole pregnancy ³². Similarly, decidual macrophages exhibit a
18 mixed M1/M2 phenotype that can secrete CCL4 ³³ during the first half of the pregnancy,
19 before switching to a M2 phenotype in the latter part of pregnancy ³⁴. Deviations in maternal
20 immune status in pregnancy have been linked with immune pathologies. Maternal exposure
21 to Th2-inducing environmental irritants enhance offspring allergic responses ³⁵, and maternal
22 IL-17a promotes autism-like behavior abnormalities in offspring in mice ³⁶. Lindehammer and
23 co-workers found an association between increased IFN- γ in maternal sera and T1D in the
24 offspring ^{18, 37-39}. Our observation of an association between M1 cytokine levels in mothers
25 and offspring T1D risk supports that a skewing towards type 1 immune responses in mothers
26 could influence offspring T1D risk. ^{38,39} The underlying mechanism behind the potential
27 association of increased maternal plasma levels of M1 cytokines, in particular CCL4, and
28 offspring T1D remains speculative. CCL4 is a CC chemokine that has affinity for the C-C
29 chemokine receptor type 5 (CCR5), and is a chemoattractant for, and activates, several
30 immune cells ⁴⁰. In the pregnant woman, there are several sources for plasma cytokines,
31 including circulating leukocytes and the placenta. Whether the cytokine pattern observed
32 during pregnancy in the maternal circulation reflect the fetal circulation is uncertain, but
33 maternal inflammation has been associated with cord blood inflammation ⁴¹. The crossing of
34 these cytokines over the placenta has not been studied in detail, although CCL4 are
35 expressed at the maternal-fetal interface ⁴². CCL2 and CCL4 are found in cord blood, with
36 higher levels in preterm infants ⁴³, so macrophages with phenotypes similar to M1
37 macrophages could be present in fetal life. Indeed, macrophages are involved in fetal

development, and recent evidence suggests that the majority of tissue macrophages in adults, at least in mice, are of fetal origin ⁴⁴. It is therefore tempting to speculate that the maternal immunological state can program fetal macrophages to a M1 phenotype causing a susceptibility to T1D development in the offspring. Such programming could be a result of maternal IFN- γ crossing the placenta or affecting fetal immune cells at the maternal-fetal interface, or involve a higher level of regulation, such as epigenetic factors, but these possibilities need to be assessed in further studies and are currently unclear.

Our findings are in accordance with studies in T1D patients which demonstrate elevated circulating levels of CCL4 ^{45, 46}, CCL2 ^{7, 45} and IFN- γ ⁸. High-risk individuals without T1D had an elevated level of CCL4 ^{9, 47} and IFN- γ ⁸, and IFN- γ levels were higher in high-risk persons than in patients with recent onset T1D⁴⁸. In experimental studies using mouse models, these cytokines are essential in initiating pancreatic inflammation. Overexpression of IFN- γ in β -cells has been shown to lead to destruction of β -cells ⁴⁹, and expression of CCL2 by β -cells leads to accumulation of macrophages in the islets ⁵⁰. Intriguingly, pancreatic macrophages are thought to be essential in T1D pathogenesis ^{51, 52}, being the first cells to infiltrate Langerhans islets ⁵³. Thus, previous studies support a mechanistic role for M1-associated cytokines in orchestrating inflammation and injury early T1D development, although experimental studies are contradictory regarding the role of CCL4 ⁵⁴.

Many of our findings are coherent with earlier literature and supports that obesity and smoking are inflammatory states. We demonstrated an association between increased GM-CSF levels and maternal smoking status, a link supported by in vitro studies ⁵⁵. Individuals with obesity in general have been shown to have higher circulating levels of CCL4 ⁵⁶, CXCL10 ⁵⁷, neopterin ⁵⁸ and KTR ⁵⁹, in line with our results in pregnant women. We also found an association of maternal IL-6 with BMI (fold difference 1.05, $p = 0.02$), which has been contradictory in previous studies ⁶⁰. Obesity is associated with increased M1 polarization ³⁷, and maternal obesity is a risk factor for offspring diabetes ¹². Although inflammation could be a potential link between maternal obesity and offspring T1D risk, the fact that the association between maternal obesity and offspring T1D was not materially changed after adjustment for immunological markers suggest that the immunological markers measured in our study did not explain the link between maternal obesity and offspring T1D. Furthermore, we found significant associations between the established T1D susceptibility SNP rs11594656 (near *IL2RA*) and soluble IL-2R α levels, supporting similar observations in non-pregnant adults ^{28, 61}, and between rs3024505, flanking the *IL10* locus and maternal circulating IL-10, consistent with a previous study ⁶². The association between rs75352297 (a SNP near CCR2 and CCR3), and CCL4 levels is somewhat surprising as the main receptor for CCL4 is CCR5. However, rs75352297 is in linkage disequilibrium with rs6808835, which has been reported to influence CCL4 levels in cerebrospinal fluid ⁶³, and is

1 in the *CCR-Like 2* gene which is close to *CCR5*. Thus, rs75352297 might be a marker for
2 haplotypes which could influence CCL4 levels, although the specific mechanisms are
3 unknown.

4 Strengths of the study include the prospective design, the large number of samples
5 tested and prospectively collected information on covariates that allowed us to statistically
6 adjust for a number of relevant factors. Weaknesses of the study include suboptimal sample
7 handling. In cord samples, marker concentrations could be influenced by a longer period
8 before separation, and an increase in neopterin levels has been reported in similar conditions
9 ⁶⁴. However, because of our prospective design, any influence of suboptimal sample handling
10 should not depend on outcome (T1D case status) and is therefore not likely to create any
11 spurious association. On the other hand, we cannot exclude type II errors. Some samples
12 underwent several freeze-thaw cycles, but our data indicate that the measured
13 concentrations are only slightly changed by repeated freeze-thaw cycles. We cannot exclude
14 the role of processes taking place in other periods of the pregnancy, local effects by
15 cytokines that are not secreted systemically, or effects exerted by cytokines restricted to the
16 fetal circulation. Reports of links between cytokine levels in newborns and T1D risk ⁶⁵
17 indicate that the cytokine patterns in the fetal circulation may hide additional cues to the
18 evolution of T1D predisposition. Although we attempted to limit the number of
19 immunological markers to test, we cannot exclude multiple testing problems.

20 In conclusion, increased mid-pregnancy maternal levels of cytokines related to
21 activated M1 macrophages, in particular CCL4, might influence offspring T1D risk, although
22 more studies are needed to confirm this association.

23

24

Conflicts of interest

Dr. Ueland is a member of the steering board of the nonprofit Foundation to Promote Research into Functional Vitamin B₁₂ Deficiency, which owns Bevital. Dr. Midttun is an employee at Bevital, which carried out the analyses on cord blood. No other potential conflict of interest relevant to this article was reported. The authors alone are responsible for the content and writing of the paper.

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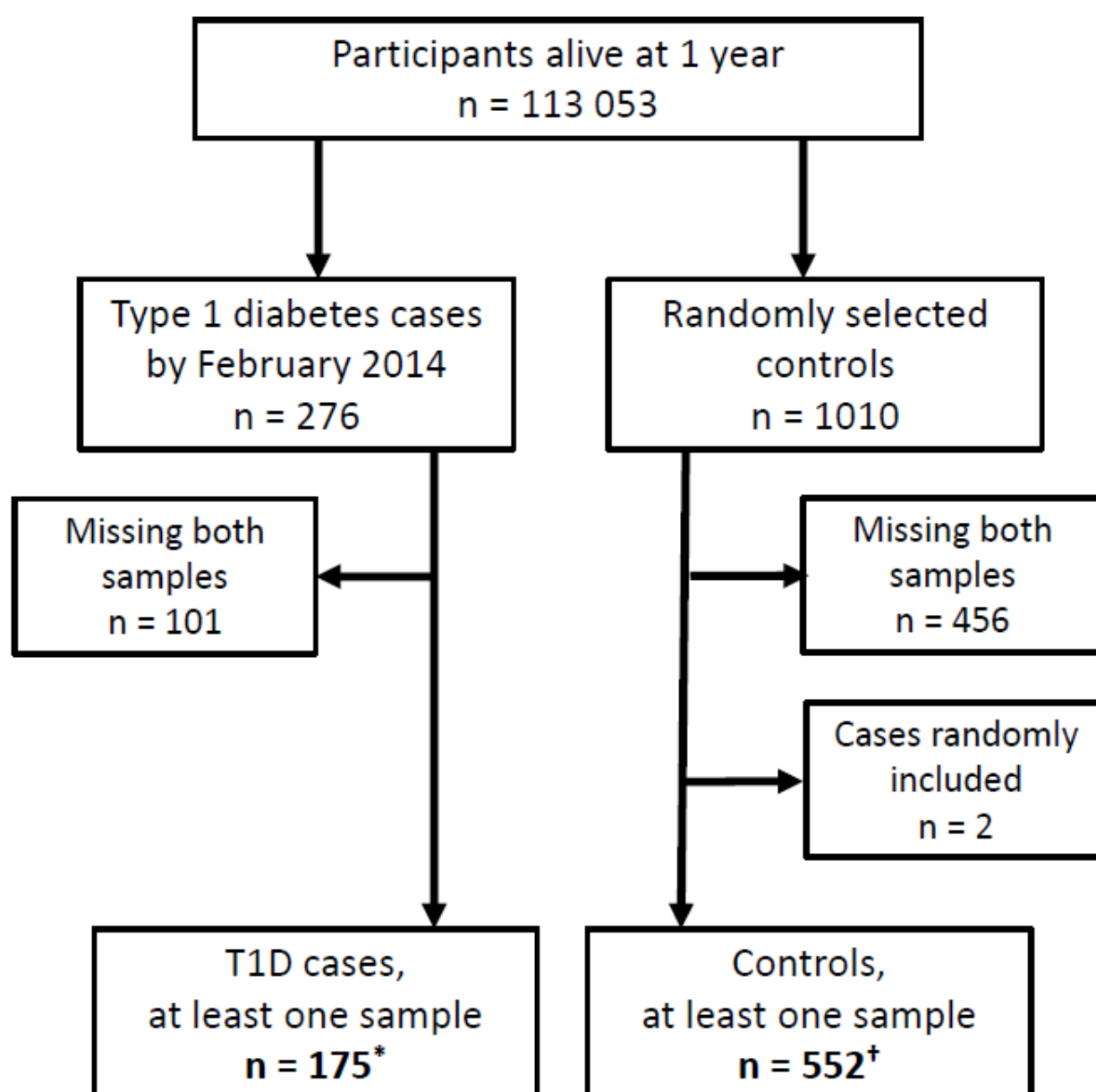
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Table I: Characteristics of the study participants

	Controls (n= 552)	Cases (n =175)
Median age (range) at end of follow-up (years)	8.6 (4.7-14.1)	9.8 (5.0-13.3)*
Female sex	272 (49.3%)	87 (49.7%)
Maternal type 1 diabetes	0 (0.0%)	7 (4.0%)
Preterm birth	19 (3.4%)	10 (5.7%)
<i>Missing data</i>	1 (0.2%)	3 (1.7%)
Birthweight (in grams)		
<2500 g	8 (1.4%)	7 (4.0%)
2500-3500 g	231 (41.9%)	66 (37.7%)
>3500-4500 g	285 (51.6%)	94 (53.7%)
>4500 g	28 (5.1%)	8 (4.6%)
Parity		
No earlier births	240 (43.5%)	86 (49.1%)
One	203 (36.8%)	53 (30.3%)
Two or more	109 (19.8%)	36 (20.6%)
Maternal age (years)		
<25	70 (12.7%)	16 (9.1%)
25-35	390 (70.6%)	134 (76.6%)
>35	92 (16.7%)	25 (14.3%)
Self-reported maternal infections in pregnancy		
None	191 (34.6%)	62 (35.4%)
One	172 (31.2%)	54 (30.9%)
Two or more	189 (34.2%)	59 (33.7%)
Maternal smoking during pregnancy		
Not during pregnancy	388 (70.3%)	127 (72.6%)
Stopped smoking prior to pregnancy	14 (2.5%)	5 (2.9%)
Smoked at any time during pregnancy	119 (21.6%)	37 (21.1%)
<i>Missing data</i>	31 (5.6%)	6 (3.4%)
Maternal pre-pregnancy BMI (kg/m²)		
<25	309 (55.6%)	76 (43.4%)
25-30	136 (24.6%)	55 (31.4%)
>30	50 (9.1%)	28 (16.0%)
<i>Missing data</i>	57 (10.3%)	16 (9.1%)
Childs HLA genotype and genetic risk score		
Protective (DQ6)	171 (31.0%)	3 (1.7%)
Baseline (any other HLA not mentioned)	112 (20.3%)	5 (2.9%)
Increased risk (≥1 copy of either DQ8 or DQ2.5)	210 (38.0%)	94 (53.7%)
High risk (DQ8/DQ2.5 heterozygote)	31 (5.6%)	72 (41.1%)
<i>Missing HLA genotype</i>	28 (5.1%)	1 (0.6%)
T1D non-HLA GRS† (median, IQR)	61.2, 47.3 – 75.2	63.2, 51.0 – 76.7
Number of freeze-thaw cycles (median, range) ‡		
Cord blood samples	3, 1 – 9	3, 1 – 7
Mid-pregnancy samples	4, 1– 10	4, 1 – 9
Caesarean section§	57 (10.3%)	34 (19.4%)

- 1 * The median age at diagnosis of T1D cases was 5.8 (range 0.7 – 13.0) years
- 2 † The genetic risk score was calculated as the number of risk alleles of SNPs associated
- 3 with T1D, weighted by their ORs (see Supplemental Table 1).
- 4 ‡ Categorized as 1, 2, 3, 4, 5, 6.3 for cord blood samples and 1, 2, 3, 4, 5, 6, 7.5 for maternal
- 5 mid-pregnancy samples. To avoid small groups, all freeze-thaw cycles >5 in cord blood and
- 6 >6 in maternal mid-pregnancy samples were categorized together at their mean value (6.3
- 7 and 7.5 for cord blood and maternal mid-pregnancy samples, respectively).
- 8 § includes unknown (n = 1), emergency (n = 53) and elective (n = 37) caesarean section

1 Figure 1: Flow chart of the participants

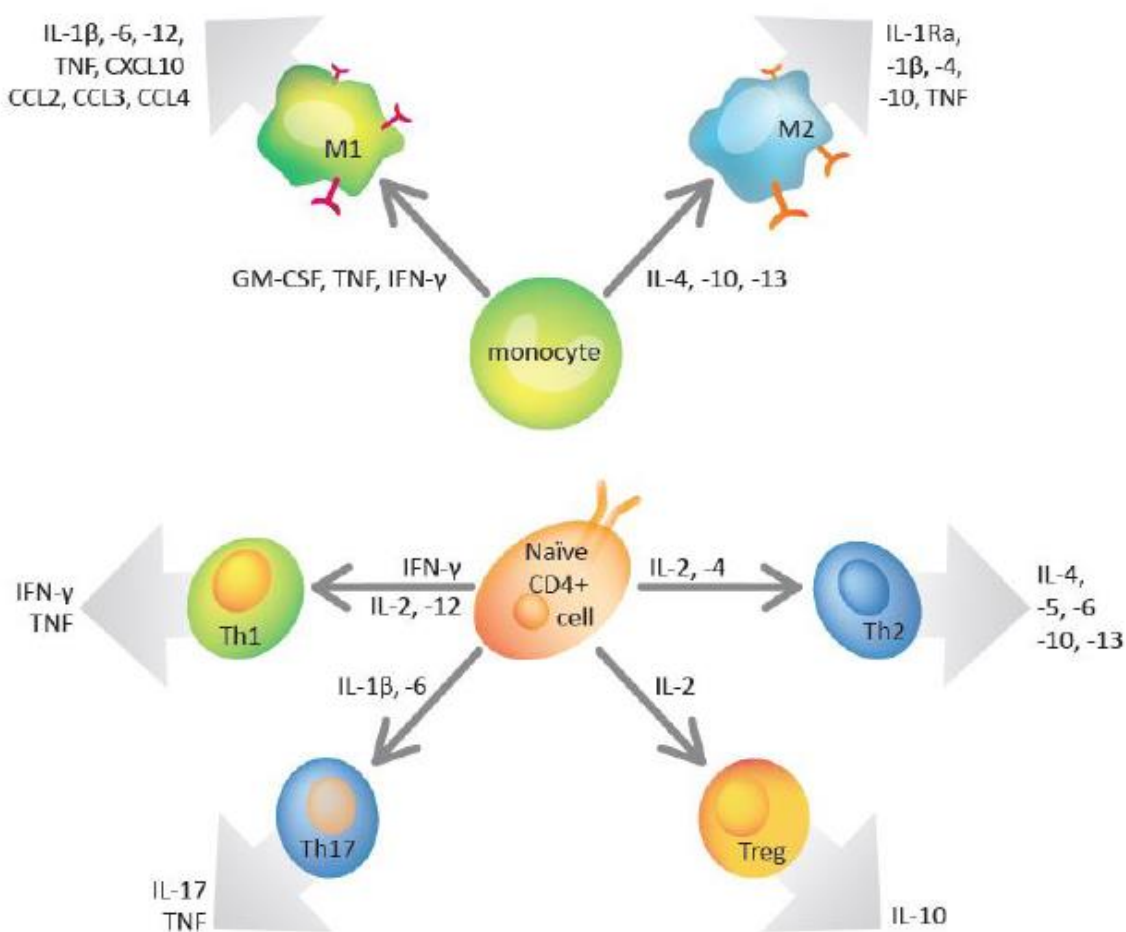


*: 150 both, 161 maternal samples, 161 neopterin and 164 KTR cord blood samples

†: 473 both, 511 maternal samples, 509 neopterin and 514 KTR cord blood samples

1 **Figure 2: Simplified overview of the stimulation by, and secretion of, the selected**
 2 **maternal immunological markers.**

A

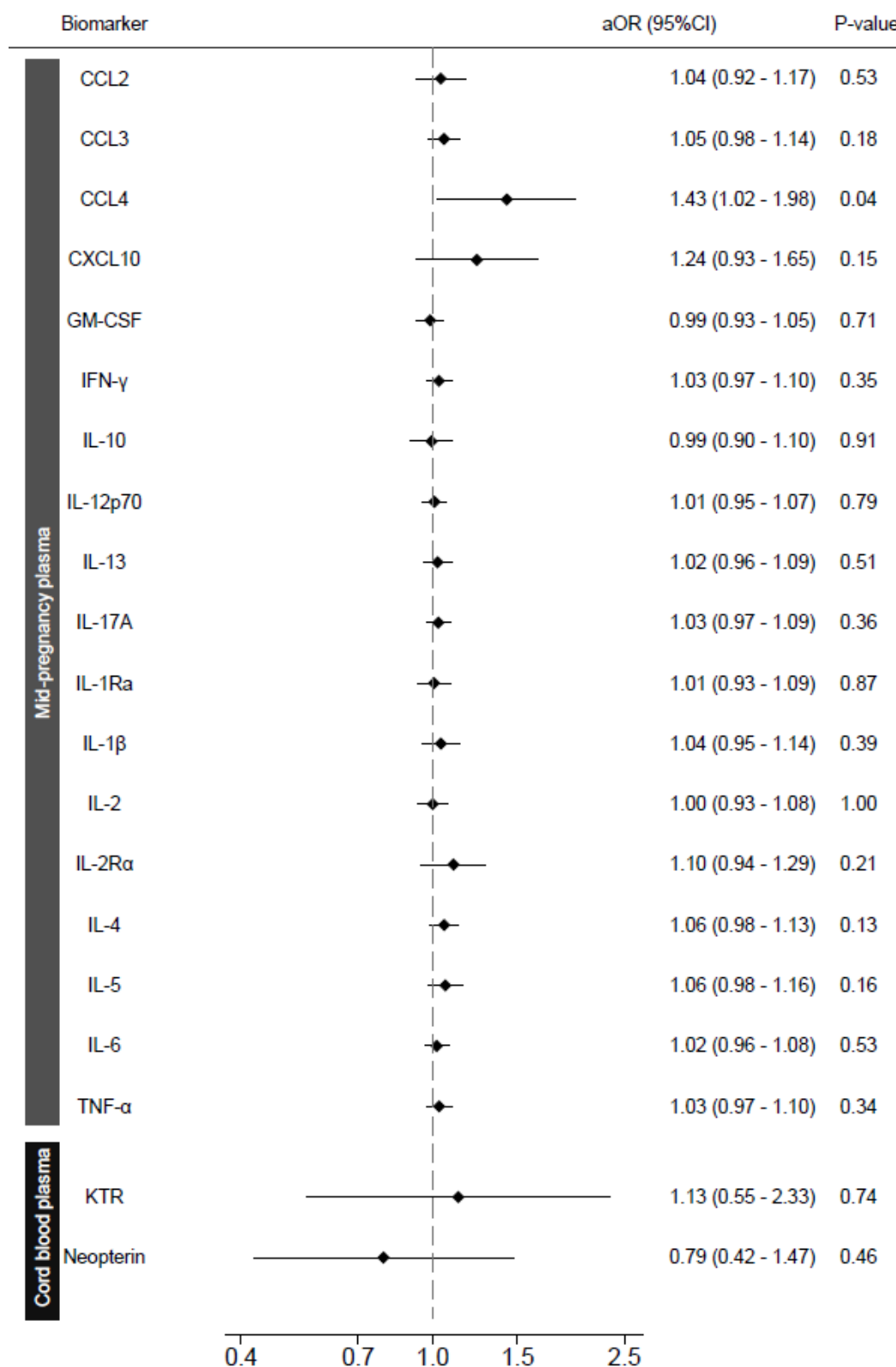


B

Pathway (cell type)	Cytokines	P value
Adaptive immune system		
Th1	IL-12, IL-2 α * (differentiation), IFN- γ and TNF- α (secretion)	0.53
Th2	IL-4 (differentiation and secreted by Th1) and IL-2 α * (differentiation), IL-5, IL-13 (secretion)	0.44
Th17	IL-6 and IL-1 β (differentiation), IL-17 (secretion)	0.23
Innate immune system		
M1	GM-CSF and IFN- γ (activation), CXCL10, CCL2, CCL3 and CCL4 (secretion)	0.02
M2	IL-4 and IL-13 (activation), IL-1 α and IL-10 (activation and secretion)	0.14

3 *IL2-R α chosen as a better marker of IL-2 activity than IL-2 itself

Figure 3: Immunological markers and their association with offspring T1D per log2 increase. A log2 increase represents a doubling. These results are adjusted for maternal pre-pregnancy BMI, child's T1D HLA risk category, gestational age at delivery, mode of delivery and year of birth. Neopterin is additionally adjusted for degree of hemolysis in cord blood.



adjusted Odds Ratio: aOR; 95% CI confidence interval: 95% CI